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Research Article

Chemical composition, antioxidant properties, and enzyme inhibitory activities of methanol extract from *Sideritis montana* subsp. *montana* using ultrasound-assisted extraction

Fatma Özlem Kargın Solmaz[1*], **Cengiz Sarikurkcu**[2]

[1]Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Afyonkarahisar Health Sciences University, Afyonkarahisar, Türkiye

[2]Department of Analytical Chemistry, Faculty of Pharmacy, Afyonkarahisar Health Sciences University, Afyonkarahisar, Türkiye

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Abstract: Natural products are valuable sources of bioactive compounds with therapeutic potential. This study investigated the chemical composition, antioxidant properties, and enzyme inhibitory activities of the methanol extract from *Sideritis montana* L. subsp. *montana* obtained via ultrasound-assisted extraction. The extraction yielded 5.37%, with a total phenolic content of 63.27 mg GAEs/g extract and a total flavonoid content of 58.32 mg REs/g extract. Chlorogenic acid (563 µg/g extract), luteolin 7-glucoside (513 µg/g extract), and hyperoside (511 µg/g extract) were the most abundant phenolics. Moderate levels of luteolin and hydroxybenzoic acids were also identified. Antioxidant activity was most pronounced in the phosphomolybdenum assay (428.52 mg TEs/g extract), followed by the CUPRAC (217.40 mg TEs/g extract) and FRAP (171.33 mg TEs/g extract) assays, demonstrating strong reducing power. Radical scavenging assays (DPPH: 122.76 mg TEs/g, ABTS: 140.41 mg TEs/g) showed moderate efficacy, while ferrous ion chelation was weak (6.62 mg EDTAEs/g extract). Enzyme inhibition assays indicated potent α-glucosidase (753.81 mg ACEs/g extract) and α-amylase (274.95 mg ACEs/g extract) inhibition, suggesting antidiabetic potential. Tyrosinase inhibition (68.56 mg KAEs/g extract) points to possible dermatological applications, though acetylcholinesterase (2.08 mg GALAEs/g extract) and butyrylcholinesterase (0.45 mg GALAEs/g extract) inhibition was minimal. The results emphasize the bioactive potential of *S. montana* subsp. *montana*. Future studies should explore its bioactivity in vivo and identify synergistic effects among its phenolic compounds to further validate its therapeutic applications.

1. INTRODUCTION

In recent years, medicinal plants have garnered significant interest due to their bioactive compounds with antioxidant potential (Grzegorczyk *et al*., 2007; Miliauskas *et al*., 2004; Mohamed *et al*., 2010). Antioxidants are known to play a crucial role in safeguarding cells from

*CONTACT: Fatma Özlem KARGIN SOLMAZ ozlem.solmaz@afsu.edu.tr Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Afyonkarahisar Health Sciences University, Afyonkarahisar, Türkiye

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oxidative stress, a condition that can lead to cellular damage (Gião *et al*., 2007). Medicinal plants are rich sources of various natural substances, including phenolic acids, flavonoids, terpenoids, vitamins, and tannins, all of which contribute to their antioxidant properties (Ai-Li & Chang-Hai, 2006; Bouayed *et al*., 2007). Notably, plants belonging to the Lamiaceae family have been widely investigated for their strong antioxidant and free radical scavenging activities (Barros *et al*., 2010; Erdemoglu *et al*., 2006).

Tyrosinase is an enzyme containing copper that catalyzes the *o*-hydroxylation of monophenols and subsequently oxidizes o-diphenols to form o-quinones (Zhou *et al*., 2017). While this process is crucial for melanin production in mammals and browning in plants and microbes, excessive melanin synthesis in human skin can lead to hyperpigmentation. Therefore, inhibiting tyrosinase activity represents a significant therapeutic approach for managing skin discoloration (Eruygur & Uçar, 2018). In a similar vein, Alzheimer's disease (AD), a chronic neurodegenerative disorder, is linked to reduced acetylcholine levels, a vital neurotransmitter in the brain. The enzymes acetylcholinesterase and butyrylcholinesterase break acetylcholine into choline and acetic acid, and their inhibition is a core strategy in AD treatment (Eruygur & Uçar, 2018; Ertaş *et al*., 2014). However, the side effects associated with synthetic inhibitors have driven the search for alternative, natural cholinesterase inhibitors. Diabetes mellitus, another complex metabolic disorder, frequently results in severe complications. Postprandial hyperglycemia, caused by glucose absorption in the digestive tract, is a major challenge in diabetic patients. Regulating blood sugar by limiting glucose uptake in the intestines and promoting its utilization in tissues offers an effective way to manage this issue. Natural compounds derived from plants are increasingly investigated for their ability to inhibit enzymes like tyrosinase and cholinesterases, offering safer and potentially more effective solutions (Eruygur & Uçar, 2018; Thilagam *et al*., 2013).

The *Sideritis* genus, a key group within the Lamiaceae family (subfamily Lamioideae), consists of approximately 150 species, primarily distributed across the Eastern and Western Mediterranean regions (de Castro & Rivera Núñez, 1994). Commonly referred to as "mountain tea," these plants are traditionally consumed as infusions with tonic, carminative, diuretic, and digestive properties (Özcan *et al*., 2001). Additionally, they are highly regarded as culinary herbs and for their use in flavor production (Palá-Paúl *et al*., 2006). In traditional medicine, *Sideritis* species are extensively employed for their anti-inflammatory, anti-ulcer, antimicrobial, cytostatic, astringent, and vulnerary effects, as well as for treating flu and stimulating circulation (Palomino *et al*., 1996). The genus is notable for its secondary metabolites, particularly diterpenoids with an ent-kaurane structure, which exhibit various bioactivities (Alessandro Venditti *et al*., 2016). Essential oils from *Sideritis* species can be categorized into three primary chemotypes: monoterpene-rich, sesquiterpene-rich, and diterpene-rich (Giuliani *et al*., 2011; Kirimer *et al*., 2004). Other bioactive components include flavonoids, phenylpropanoid and phenylethanoid glycosides, and iridoids (Alessandro Venditti *et al*., 2016; Venditti *et al*., 2013).

This study aims to comprehensively evaluate the chemical composition, antioxidant potential, and enzyme inhibitory activities of the methanol extract obtained from *Sideritis montana* L. subsp. *montana* using ultrasound-assisted extraction. By quantifying the total phenolic and flavonoid contents and identifying the key phytochemicals through advanced chromatographic techniques, we seek to elucidate the bioactive components responsible for the plant's biological properties. Additionally, the investigation of antioxidant and enzyme inhibitory activities will provide critical insights into the therapeutic potential of this subspecies. This research not only contributes to the growing body of knowledge on *Sideritis* species but also highlights the importance of innovative extraction techniques in enhancing the discovery of natural compounds with promising pharmacological applications.

2. MATERIAL and METHODS

2.1. Plant Material

Aerial parts of *S. montana* subsp. *montana* were harvested on May 17, 2022, from Nebiler village, Kavaklıdere-Muğla, Türkiye (elevation: 1060 m, coordinates: 37°27'35"N, 28°24'60"E). The species was identified by Dr. Olcay Ceylan, and a voucher specimen (O.2154) was deposited in the Herbarium of the Department of Biology, Muğla Sıtkı Koçman University, Türkiye. The collected material was air-dried in shaded conditions for several weeks and ground into a fine powder using a laboratory grinder.

2.2. Methanol Extraction

Ultrasound-assisted extraction (UAE) was employed to prepare the methanol extract, using a sample-to-solvent ratio of 1:20. The process was performed in a sonication bath for one hour. After extraction, the methanol was removed under reduced pressure with a rotary evaporator, and the obtained extract was stored at 4°C for further analysis.

2.3. Determination of Chemical Composition

The total flavonoid content (TFC) was quantified using the aluminum chloride method, while the total phenolic content (TPC) was assessed with Folin-Ciocalteu reagent. Results were expressed as rutin equivalents (REs) and gallic acid equivalents (GAEs), respectively, following protocols described by Sarikurkcu *et al*. (2013). The phytochemical composition was further analyzed using a validated chromatographic method reported by Cittan and Çelik (2018). Detailed methodological parameters are provided in the supplementary material.

2.4. Biological Activity

Antioxidant activity was evaluated using multiple methods (Apak *et al*., 2006; Kocak *et al*., 2016; Sarikurkcu *et al*., 2020; Tepe *et al*., 2011; Zengin *et al*., 2017;), while enzyme inhibitory activities were assessed based on the protocols of Ozer *et al*. (2018). Full descriptions of the methods are presented in the supplementary file.

2.5. Statistical Analysis

All results are expressed as means ± SD, based on three independent replicates)

3. RESULTS and DISCUSSION

3.1. Chemical Composition

The methanol extract of *S. montana* subsp. *montana*, obtained using ultrasound-assisted extraction, exhibited an extraction yield of 5.37%. The total phenolic content of the extract was determined to be 63.27 mg GAEs/g extract, while the total flavonoid content was measured as 58.32 mg REs/g extract (Table 1). These findings indicate that the extract possesses a relatively high concentration of phenolic and flavonoid compounds, which may contribute to its potential bioactivity. All values represent the mean ± standard deviation (SD) of three independent replicates, with phenolic and flavonoid contents expressed in terms of gallic acid and rutin equivalents, respectively.

Table 1. Extraction yield, total phenolic and flavonoid contents of the methanol extract from *S. montana* subsp. *montana*.

Assays	Methanol extract
Extraction yield (%)	5.37
Total flavonoids (mg REs/g extract)	58.32±0.05
Total phenolics (mg GAEs/g extract)	63.27±2.17

Values expressed are means ± SD of three parallel measurements. REs and GAEs rutin and gallic acid equivalents.

The chemical composition analysis of the methanol extract from *S. montana* subsp. *montana*, obtained through ultrasound-assisted extraction, revealed the presence of various phenolic

compounds in differing concentrations (Table 2). Among the quantified compounds, chlorogenic acid was identified as the most abundant (563 µg/g extract), followed by luteolin 7-glucoside (513 µg/g extract) and hyperoside (511 µg/g extract). Moderate levels of luteolin (41.1 µg/g extract), 4-hydroxybenzoic acid (40.4 µg/g extract), and 3-hydroxybenzoic acid (39.3 µg/g extract) were also detected. Additionally, apigenin 7-glucoside, *p*-coumaric acid, and vanillin were present in concentrations of 39.3, 33.0, and 20.1 µg/g extract, respectively.

Other phenolic compounds, such as verbascoside, 2,5-dihydroxybenzoic acid, and protocatechuic acid, were identified in smaller amounts. Flavonoids like and apigenin were also detected, albeit at lower concentrations. Compounds such as ferulic acid, pinoresinol, and syringic acid were present in trace amounts. In contrast, certain compounds, including 3,4-dihydroxyphenylacetic acid, 2-hydroxycinnamic acid, and rosmarinic acid, were not detected in the extract.

The chemical composition of the methanol extract obtained via ultrasound-assisted extraction from *S. montana* subsp. *montana* presents novel and significant findings when compared to the previously reported data in the literature.

Table 2. Concentration of selected phenolic compounds in the methanol extract from *S. montana* subsp. *montana*.

Compounds	Concentration (µg/g extract)
Chlorogenic acid	563±25
Luteolin 7-glucoside	513±6
Hyperoside	511±3
Luteolin	41.1±0.7
4-Hydroxybenzoic acid	40.4±0.5
3-Hydroxybenzoic acid	39.3±1.7
Apigenin 7-glucoside	39.3±3.6
p-Coumaric acid	33.0±0.3
Vanillin	20.1±3.2
Verbascoside	18.4±0.1
2,5-Dihydroxybenzoic acid	18.2±0.3
Protocatechuic acid	17.2±0.6
Quercetin	13.8±0.1
Apigenin	11.0±0.3
Ferulic acid	9.54±0.45
Pinoresinol	6.84±0.33
Caffeic acid	4.82±0.74
Syringic acid	5.26±0.02
Hesperidin	4.88±0.32
Gallic acid	1.74±0.01
Eriodictyol	0.56±0.03
(-)-Epicatechin	0.22±0.01
3,4-Dihydroxyphenylacetic acid	nd
2-Hydroxycinnamic acid	nd
(+)-Catechin	nd
Pyrocatechol	nd
Sinapic acid	nd
Taxifolin	nd
Rosmarinic acid	nd
Kaempferol	nd

Values expressed are means ± SD of three parallel measurements.

The current study reports a total phenolic content and a total flavonoid content, indicative of high phenolic and flavonoid concentrations. These values surpass the phenolic and flavonoid content observed in earlier studies that focus on ethanolic or seed extracts of *S. montana* subsp. *montana* (e.g., Emre *et al*. (2011)). This discrepancy may stem from differences in extraction techniques and solvent systems, as ultrasound-assisted extraction is known to enhance the recovery of bioactive compounds.

The current analysis identified chlorogenic acid, luteolin 7-glucoside, and hyperoside as the predominant compounds. While flavonoids and caffeoylquinic derivatives were previously noted in the work of A. Venditti *et al*. (2016), the quantified levels of these compounds, especially chlorogenic acid, are significantly higher in the present study. This may suggest a potential geographical or ecological variability influencing the plant's metabolomic profile.

In contrast to earlier studies that highlighted flavonoids such as morin and catechin as dominant (Emre *et al*., 2011), the current findings emphasize luteolin and its glycosides, hyperoside, and apigenin 7-glucoside as key flavonoids. These differences could be attributed to variations in plant part usage, solvent polarity, or specific environmental factors influencing secondary metabolite biosynthesis.

The presence of 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, vanillin, and *p*-coumaric acid in the current study constitutes a new record for *S. montana* subsp. *montana*. These phenolic acids and aromatic compounds have not been previously reported for this subspecies in the literature, highlighting their novelty as phytochemical constituents. These findings contribute to the chemotaxonomic characterization of the species and broaden the phytochemical spectrum associated with the genus *Sideritis*.

The predominance of chlorogenic acid and specific flavonoids aligns with the chemotaxonomic markers of the *Sideritis* genus, as discussed by Kilic (2014). However, the identification of unique phenolic acids and aromatic compounds, such as vanillin and *p*-coumaric acid, differentiates *S. montana* subsp. *montana* from closely related taxa, supporting its distinct chemotaxonomic status.

The novel compounds identified in this study, combined with the high phenolic and flavonoid content, underscore the bioactive potential of *S. montana* subsp. *montana*. The presence of compounds like vanillin and *p*-coumaric acid, which are known for their antioxidant and antimicrobial properties, suggests potential applications in pharmaceutical and nutraceutical formulations.

The current findings not only align with the general chemical characteristics of *Sideritis* species but also extend the phytochemical knowledge of *S. montana* subsp. *montana* by reporting new compounds and higher concentrations of known constituents. These results underscore the importance of using advanced extraction and analysis methods for uncovering the full metabolomic potential of medicinal plants.

3.2. Antioxidant Activity

The antioxidant potential of the methanol extract from *S. montana* subsp. *montana*, obtained via ultrasound-assisted extraction, was evaluated using multiple in vitro assays, with results indicating notable activity across various mechanisms (Table 3). The highest antioxidant activity was observed in the phosphomolybdenum assay, with a total antioxidant capacity of 428.52 mg TEs/g extract, reflecting the extract's strong ability to reduce molybdenum(VI) to molybdenum(V). This was followed by significant activity in the CUPRAC (217.40 mg TEs/g extract) and FRAP (171.33 mg TEs/g extract) assays, both of which assess reducing power and suggest the presence of compounds capable of donating electrons to neutralize reactive species. The radical scavenging activities, evaluated using DPPH and ABTS assays, were measured at 122.76 and 140.41 mg TEs/g extract, respectively, indicating the extract's moderate efficiency in neutralizing free radicals. In contrast, the ferrous ion chelating activity was relatively low, at

6.62 mg EDTAEs/g extract, suggesting a limited ability to bind metal ions and inhibit metal-catalyzed oxidative reactions.

Table 3. Antioxidant activity of the methanol extract from *S. montana* subsp. *montana*.

Assays	Activity
Phosphomolybdenum (mg TEs/ g extract)	428.52±7.81
CUPRAC reducing power (mg TEs/ g extract)	217.40±14.54
FRAP reducing power (mg TEs/ g extract)	171.33±2.13
DPPH radical scavenging (mg TEs/ g extract)	122.76±2.42
ABTS cation radical scavenging (mg TEs/ g extract)	140.41±6.69
Ferrous ion chelating (mg EDTAEs/ g extract)	6.62±0.59

Values expressed are means ± SD of three parallel measurements. TEs and EDTAEs, trolox and ethylenediaminetetraacetic acid (disodium salt) equivalents, respectively.

When the antioxidant activity results are considered alongside the chemical composition data, the high activity observed in the phosphomolybdenum, CUPRAC, and FRAP assays can be attributed to the extract's abundant phenolic and flavonoid content, particularly chlorogenic acid, luteolin 7-glucoside, and hyperoside. These compounds are well-documented for their electron-donating properties, which likely contribute to the extract's robust reducing power and overall antioxidant capacity (Gao *et al*., 2019; Maatouk *et al*., 2017; Park *et al*., 2016; Sato *et al*., 2011; Wu, 2007). The moderate radical scavenging activities observed in the DPPH and ABTS assays may also be linked to these phenolics, as well as other bioactive constituents such as luteolin and *p*-coumaric acid, which are known to quench free radicals through hydrogen atom transfer or single electron transfer mechanisms (Gökbulut *et al*., 2012; Kiliç & Yeşiloğlu, 2013; Masek *et al*., 2016; Tian *et al*., 2021).

The relatively low ferrous ion chelating activity suggests that metal chelation is not a primary mechanism of action for this extract, which aligns with the chemical composition data indicating lower concentrations of compounds like quercetin and apigenin, which possess chelating properties. Overall, the methanol extract's antioxidant profile underscores the prominent role of its phenolic constituents, particularly chlorogenic acid, luteolin 7-glucoside, and hyperoside, in contributing to its bioactivity.

3.3 Enzyme Inhibitory Activity

The enzyme inhibition potential of the methanol extract from *S. montana* subsp. *montana* was evaluated through multiple assays targeting key enzymes involved in various physiological and pathological processes (Table 4).

The extract demonstrated the highest inhibitory activity against α-glucosidase, with a value of 753.81 mg ACEs/g extract. This suggests a strong potential for managing hyperglycemia through the modulation of carbohydrate metabolism. Similarly, significant inhibition was observed for α-amylase, with an activity of 274.95 mg ACEs/g extract, indicating its complementary role in reducing postprandial glucose levels.

Table 4. Enzyme inhibition activity of the methanol extract from *S. montana* subsp. *montana*.

Assays	Activity
Acetylcholinesterase inhibition (mg GALAEs/g extract)	2.08±0.03
Butyrylcholinesterase inhibition (mg GALAEs/g extract)	0.45±0.01
Tyrosinase Inhibition (mg KAEs/g extract)	68.56±0.26
α-Amylase inhibition (mg ACEs/g extract)	274.95±3.06
α-Glucosidase inhibition (mg ACEs/g extract)	753.81±8.87

Values expressed are means ± SD of three parallel measurements. ACEs, GALAEs and KAEs mean acarbose, galanthamine and kojic acid equivalents, respectively.

The tyrosinase inhibition assay revealed moderate activity, with a value of 68.56 mg KAEs/g extract, suggesting potential applicability in dermatological formulations targeting hyperpigmentation. In contrast, the extract exhibited relatively low inhibition against acetylcholinesterase (2.08 mg GALAEs/g extract) and butyrylcholinesterase (0.45 mg GALAEs/g extract), indicating limited efficacy in addressing cholinesterase-associated disorders, such as Alzheimer's disease.

When these findings are considered alongside the chemical composition data, the observed enzyme inhibitory activities can be attributed to the high phenolic and flavonoid content of the extract. The strong α-glucosidase and α-amylase inhibitory activities may be linked to the abundance of chlorogenic acid, which has been widely reported to inhibit carbohydrate-hydrolyzing enzymes (Oboh *et al*., 2015; Wang *et al*., 2022; Zheng *et al*., 2020). Additionally, compounds such as hyperoside (Shen *et al*., 2023), and *p*-coumaric acid (Huang *et al*., 2023; Khan *et al*., 2022) may contribute to these effects through their known enzyme-modulating properties.

The moderate tyrosinase inhibition activity is likely influenced by phenolics such as vanillin (Ashraf *et al*., 2015; Rescigno *et al*., 2011) and apigenin 7-glucoside (Bouzaiene *et al*., 2016; Sezen Karaoğlan *et al*., 2023), both of which are recognized for their ability to interfere with melanin biosynthesis. The relatively weak cholinesterase inhibition observed in the study may reflect the low concentrations of specific compounds, such as quercetin and apigenin, that are more commonly associated with acetylcholinesterase and butyrylcholinesterase inhibition (Islam *et al*., 2021; Orhan, 2021).

When combined with the previously reported antioxidant activity of the extract, these results suggest that the bioactive potential of *S. montana* subsp. *montana* methanol extract arises from the synergistic effects of its phenolic constituents. The pronounced enzyme inhibition against α-glucosidase and α-amylase highlights its potential for managing oxidative stress and metabolic disorders, particularly diabetes. The contributions of chlorogenic acid, luteolin derivatives, and other phenolics underscore the importance of these compounds in defining the extract's biofunctional properties.

4. CONCLUSION

The findings of this study provide substantial evidence for the bioactive potential of the methanol extract derived from *S. montana* subsp. *montana* through ultrasound-assisted extraction. The extract’s high content of phenolic and flavonoid compounds underscores its relevance as a source of antioxidant and enzyme-inhibitory agents. Among the phenolic constituents, chlorogenic acid, luteolin 7-glucoside, and hyperoside were identified as the predominant compounds, which likely play a critical role in the observed bioactivities.

The extract exhibited pronounced antioxidant activity, particularly in the phosphomolybdenum assay, demonstrating its capacity to neutralize reactive oxygen species. This was further supported by notable CUPRAC and FRAP reducing power, which reflect the presence of potent electron-donating molecules. Moderate radical scavenging activity in the DPPH and ABTS assays and limited ferrous ion chelating ability suggest a multifaceted antioxidant mechanism, where electron transfer predominates over metal chelation. The chemical composition analysis reinforces these results, as the identified phenolics, especially chlorogenic acid and flavonoid derivatives, are well-documented for their antioxidant properties.

In terms of enzyme inhibition, the extract showed significant activity against carbohydrate-hydrolyzing enzymes, particularly α-glucosidase and α-amylase, indicating strong potential for managing hyperglycemia and diabetes-related oxidative stress. Moderate tyrosinase inhibition suggests additional applications in dermatology, particularly for hyperpigmentation disorders. However, the extract demonstrated limited cholinesterase inhibitory activity, which may diminish its therapeutic applicability in neurodegenerative diseases like Alzheimer's.

Despite these promising results, certain limitations should be acknowledged. While the study

highlights the extract's bioactive potential, the exact synergistic or individual contributions of the phenolic compounds remain unclear. Advanced studies, such as bioactivity-guided fractionation or molecular docking, could elucidate the mechanisms underlying these effects. Additionally, in vivo investigations are necessary to validate the efficacy and safety of the extract under physiological conditions, as in vitro assays may not fully represent its behavior in complex biological systems.

Future research should also explore the optimization of extraction parameters to further enhance the yield and activity of bioactive compounds. Comparative studies with other extraction techniques and solvents could provide insights into the efficiency and sustainability of ultrasound-assisted extraction for *S. montana* subsp. *montana*. Finally, assessing the stability of the bioactive components under storage and processing conditions would be critical for potential industrial or pharmaceutical applications.

In conclusion, the methanol extract of *S. montana* subsp. *montana* holds significant promise as a source of natural antioxidants and enzyme inhibitors. Its chemical richness and multifunctional bioactivities highlight its potential for therapeutic and nutraceutical applications, warranting further exploration to fully realize its benefits.

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Authorship Contribution Statement

Fatma Özlem Kargın Solmaz contributed to the experimental studies, performed antioxidant and enzyme inhibition activity experiments, preparation of manuscript and proofreading; **Cengiz Sarikurkcu** provided the plant material used, participated in phytochemical analysis and the writing and proofreading.

Orcid

Fatma Özlem Kargın Solmaz https://orcid.org/0000-0002-3908-3371
Cengiz Sarikurkcu https://orcid.org/0000-0001-5094-2520

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